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A GAC biofilm reactor for the continuous degradation of 4-chlorophenol: treatment efficiency and microbial analysis

Abstract Using a continuous enrichment technique, a bacterial consortium capable of degrading 4-chlorophenol (4-CP) was obtained from the rhizosphere of *Phragmites australis*. A granular activated carbon (GAC) biofilm reactor was established using this consortium, and the degradation of 4-CP was investigated under continuous flow operation using a feed of 20–50 mg l^{-1} with a hydraulic residence time of 17 min over a 6-month period. Chloride liberation occurred throughout the operation, and the reactor had 4-CP removal efficiencies of 69–100%. Periods of lower performance were attributed to clogging of the column with biomass and the formation of channels. Subsequently, the immobilized biofilm was subjected to a starvation period of 5 months, after which its degradative capacity was still maintained. The microbial consortium was characterized during the continuous flow experiment and dynamic population changes were observed throughout. One isolate recovered from the biofilm was shown to be capable of degrading 4-CP as a sole carbon and energy source.

Introduction

Chlorinated organic compounds form one of the most important groups of xenobiotic chemicals that enter the environment. These include some of the most useful and economically important chemicals available to industry and agriculture, being extensively used as herbicides, insecticides, fungicides, heat transfer media, insulators and lubricants (Keith and Telliard 1979; Barbash and Roberts 1996). Due to their extensive use, these compounds are common soil and water contaminants (Mason 1991; Chapelle 1993). Chloroaromatic compounds are susceptible to biodegradation, and aerobic degradation often involves their mineralization to innocuous products, thereby making the utilization of these reactions especially attractive in remediation applications. It has been reported that 4-chlorophenol (4-CP) can be partially or completely degraded aerobically by several bacteria including *Pseudomonas* (Knackmuss and Hellwig 1978), *Azotobacter* (Wieser et al. 1994), *Alcaligenes* (Hill et al. 1996), and *Rhodococcus* (Finkel'shtein et al 2000), and via different biodegradation pathways (Bae et al. 1996; Hollender et al. 1997).

Biological waste treatment techniques for water and effluents contaminated with chlorinated organic compounds often include the utilization of biofilm reactors. Adsorption and biological treatment are two common approaches used to treat such compounds (Jonge et al. 1996; Caldeira et al. 1999). Granular activated carbon (GAC) biofilm reactors can combine these two features; the adsorptive capacity and irregular shape of GAC particles provide niches for bacterial colonization protected from high fluid forces (Christensen and Characklis 1990), while the variety of functional groups on the surface can enhance the attachment of microorganisms (Weber et al. 1979). In these systems, biomass is active even at very low concentrations of target organic chemicals, making it less sensitive to the presence of toxic and inhibitory materials and more resistant to shock loading of toxics than dispersed growth systems (Lee et al. 1994; Fauzi 1995; Shi et al. 1995). In the fermentation field, biofilm systems have also been shown to lead to increased bacterial lactic acid productivities, while protecting bacteria from the effect of antibiotics used to subdue yeast contaminations (Velásquez et al., 2001). The continuous removal of pollutants in biofilm reactors has been reported by several authors. Feakin et al.(1995) used a GAC fixed bed reactor for the treatment of 1–10 µg l^{-1} atrazine and simazine in surface water and achieved removal efficiencies of 53% and 58%, respectively. Massol-Deya et al. (1995) reported a

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98% reduction of a 3 mg l^{-1} influent concentration of toluene in a fluidized bed reactor-fed toluene-amended groundwater. Similarly, treatment of groundwater contaminated with chlorobenzene at concentrations up to 170 mg l^{-1} in a GAC fluidized-bed reactor was achieved with more than 99% efficiency (Klecka et al. 1996). Compact matrixes, based on wheat husk and wheat bran, have been successfully used to immobilize the fungus *Coriolus versicolor* to treat effluents contaminated with dichlorophenol and pentachlorophenol at 50 mg l^{-1}, for which removal rates of 75–80% and 100%, respectively, were observed within 24 h of batch cultures (Ullah et al. 2000).

The present report extends our previous research on GAC biofilms (Caldeira et al. 1999) by evaluating 4-CP removal in a continuous flow bioreactor, in order to determine whether steady-state conditions could be achieved, and by analysing the robustness of the system to withstand long periods of contaminant starvation. Such reactor systems closely mimic those most commonly deployed for effluent and water biotreatment, which often face variations in concentrations of the contaminant feed. The efficiency of the GAC biofilm reactor to continuously biodegrade 4-CP from an aqueous feed, supplied at different concentrations over a period of 6 months, and the stability of the degrading activity after a prolonged period of starvation are discussed. In addition, microbiological characterization of the biofilm bacteria is described.

Materials and methods

Preparation of the bacterial inoculum

The bacteria used in this study were isolated from soil samples collected from the rhizosphere of the reed *Phragmites australis*, obtained from a chemically contaminated industrial site in Northern Portugal (Estarreja), which has received discharges of liquid effluents for over 50 years. The bacteria were recovered from the soil by a dispersion and differential centrifugation technique based on that described by Hopkins et al. (1991). Rhizosphere soil samples (10 g) were blended mechanically with 10 ml sodium cholate solution (0.1% w/v) for 1 min, after which 10 ml sodium cholate solution, 10 g chelating resin (sodium form of iminodiacetic acid) and approximately 30 glass beads (~5 mm diameter) were added. The resultant preparation was shaken for 2 h at 5°C and centrifuged at 500 g for 2 min. The supernatant (a1) was kept for subsequent inoculum formulation. The same procedure was used to obtain a further series of supernatants by successively extracting the soil pellet with the following series of solutions: 10 ml Tris buffer pH 7.4 (a2), 30 ml sodium cholate solution followed by ultrasonication for 1 min (b1), 10 ml Tris buffer (b2), and twice 40 ml of distilled water (c1+c2). The final supernatants consisted of: A (a1+a2), B (b1+b2) and C (c1+c2). A mixture of supernatants A (5 ml), B (7.5 ml), and C (10 ml) was used to inoculate a batch flask containing 300 ml of minimal salts medium (Caldeira el al. 1999). This composite preparation was used as the inoculum for biofilm reactor studies.

Biofilm development in a packed bed GAC column

The composite bacterial preparation obtained from the rhizosphere soil samples was used to inoculate a tubular glass column (26 cm×2.6 cm), packed with 15 g of thermally activated commercial GAC (12–18 mesh). Glass wool was placed in the bottom of the column to hold the GAC in place. The reactor was maintained at room temperature and operated in a downflow mode. The inoculum was recirculated through the packed column for 5 days to allow colonization of the support material. The decrease (>90%) in the optical density of the circulating inoculum was used as an indication of biomass loading onto the GAC column. Thereafter, an enrichment of 4-CP-degrading bacteria proceeded over a 50 day period. During this regime, the column was periodically supplied (1–2 times per week) with 300 ml minimal salts medium supplemented with 4-CP and phenol (10 mg l^{-1} each), which was recirculated through the column with a hydraulic residence time (HRT) of 25 min. The biofilm activity was monitored by chloride release due to the biodegradation of 4-CP. After 30 days of biofilm enrichment, the bacteria attached to the GAC were enumerated. The biomass was extracted by shearing duplicate samples (1 g GAC particles) on a vortexing machine operating at maximum speed for 30 s. Diluted suspensions (0.1 ml) were plated onto nutrient agar (NA) and minimal agar supplemented with 4-CP.

Continuous operation of the biofilm reactor

Aerated minimal medium containing 4-CP was continuously passed through the GAC reactor, during a 6-month period, with an HRT of 17 min, based on the GAC bed volume of 33 cm^3 used for the experiments. Aeration was supplied to the feeding vessel by a compressor working at 0.6 bar. Air was filtered (Nalgene SFCA 0.2 µm) before entering the vessel, and was fed into the mineral medium by means of a submerged silicone tube (internal diameter 2.8 mm, 0.8 mm thick, approximately 0.75 m length). At different stages, the 4-CP concentration in the feed was varied as follows: on days 0–51, 52–87, 88–113, 114–166 and 167–200, 4-CP concentrations in the feed were 50 mg l^{-1}, 25 mg l^{-1}, 0 mg l^{-1}, 25 mg l^{-1} and 20 mg l^{-1}, respectively. Chlorophenol and chloride concentrations in the column effluent were analysed as described previously (Caldeira et al. 1999); the presence of phenol could be detected using the same analytical procedure as for 4-CP, which was verified by analysing the separation of standard mixtures of 4-CP and phenol. Bacteria eluted from the biofilm column were enumerated every month. Effluent samples were diluted in 0.85% (w/v) NaCl solution, and 0.1 ml of appropriate dilutions were spread plated onto NA and minimal salts agar supplemented with 4-CP.

Reactor operation under starvation conditions

After 6 months of continuous operation, the biofilm reactor was starved of 4-CP and the liquid flow suspended for a further 5 months. After that period the column was operated again under a recirculating regime (9 days), during which the column was fed with 2 l of minimal salts medium supplemented with 4-CP (50 mg l^{-1}, on days 0 and 4), with an HRT of 17 min. This recirculating mode was followed by continuous operation, during which the column was fed with 4-CP (50 mg l^{-1}) with the same retention time. The column was maintained at room temperature.

Biofilm microbial community

Bacteria attached to the GAC were enumerated and characterized at different stages of bioreactor operation: at the beginning (day 0), in the middle (day 90) and at the end (day 200) of the continuous experiment; bacteria were also enumerated after the starvation period. GAC biomass loading (CFUs g^{-1} GAC) was determined after extracting the biomass as described above. Samples of approximately 0.5–1 g were taken in duplicate, collected from the inner middle section of the bed; after each sampling the amount of GAC extracted was replaced with fresh GAC. The bacterial suspensions were spread plated onto NA and minimal salts agar supplemented with 4-CP and, at the same time, used as a 20% inoculum for batch flasks containing 30 ml of minimal salts medium

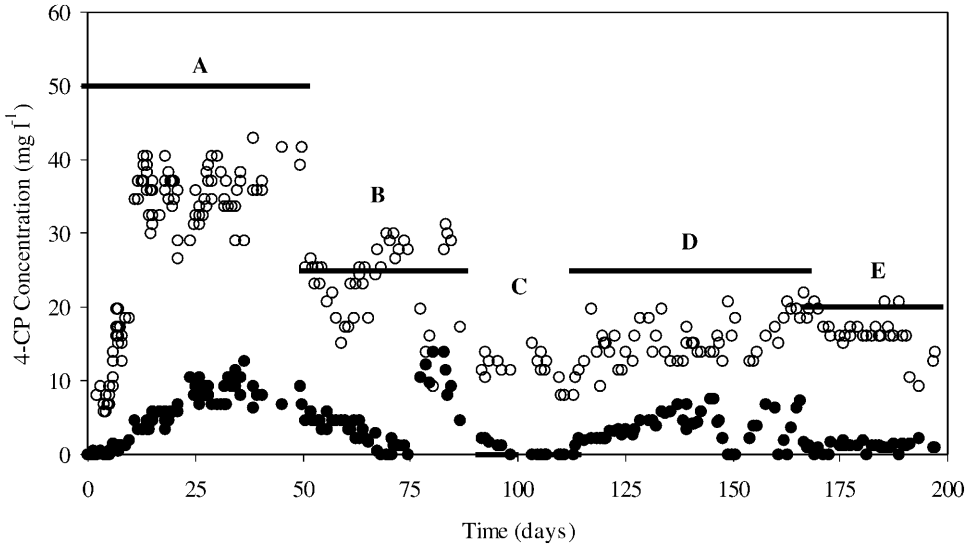

Fig. 1 Degradation of 4-chlorophenol (4-CP) in the biofilm column during continuous mode operation. *Horizontal bars* 4-CP feed concentrations during open mode operation during days *A* 0–51, *B* 52–87, *C* 88–113, *D* 114–166, *E* 167–200. *Open circles* 4-CP degraded based on chloride release, *black circles* 4-CP detected in the column effluent

supplemented with 4-CP (50 mg l^{-1}) in order to verify 4-CP biodegradation.

Bacteria recovered from the GAC matrix were purified by repeated subculturing on NA and minimal salts agar supplemented with 4-CP. A preliminary characterization was based on colony and cell morphology, presence or absence of cytochrome *c* oxidase and Gram staining. Gram-negative isolates were identified using the API 20 NE system (Biomérieux).

All microorganisms recovered from the GAC were analysed for their individual capacities to degrade 4-CP. Each colonial type was suspended in 10 ml of minimal salts medium supplemented with 25 mg l^{-1} 4-CP as the sole carbon and energy source. Cultures were grown aerobically in 100 ml flasks incubated at 25°C on a rotatory shaker (Julabo-SW-20C) at 100 rpm. Growth was monitored by measuring the optical density of the cultures at 600 nm. Biodegradation of 4-CP was measured by chloride release to the medium; residual 4-CP concentration was also determined. When growth on 4-CP was evidenced by an increase in optical density and by liberation of chloride, samples of culture were plated on NA in order to verify whether single species were present. When pure colonies were obtained, this procedure was repeated several times.

Reagents

All chemicals were of the highest purity grade available (Sigma, St. Louis, USA; Aldrich, Dorset, UK; Merck, Darmstadt, Germany). GAC (12–18 mesh) was obtained from BDH, Dorset, UK. Prior to use, GAC was washed several times with deionized water to remove carbon fines, dried in an oven at 105°C, and sterilized by autoclaving.

Results

Establishment of the 4-CP degrading biofilm

After inoculating the reactor and operating it in a closed recirculating flow mode, biodegradation of 4-CP was observed after 3 weeks as the accumulation of chloride ion in the recirculating vessel. During the subsequent 1-month period, stoichiometric release of chloride indicated that, under this recirculation mode of operation, all the supplied 4-CP could be dechlorinated. Thereafter, the column was maintained for a further 2 months by recirculating minimal medium, supplemented weekly with 4-CP.

Biodegradation of 4-CP in the continuous flow biofilm reactor

Following the establishment of an active 4-CP degrading biofilm, the performance of the reactor for the continuous removal of 4-CP was evaluated over a 6-month period. Chloride and 4-CP in the column outlet were measured The presence of phenol was also investigated, but this compound was never found in the column outflow. During the first 51 days, 4-CP was fed to the column with a concentration of 50 mg l^{-1} (Fig. 1, A). Initially (days 0–5), 4-CP was not detected in the reactor effluent, but thereafter (days 6–36) its concentration increased in the outlet, reaching a mean concentration of 8.9±1.5 mg l^{-1}. Chloride release was observed during this period, increasing steadily until day 14, and reaching an average concentration thereafter of 35.1±3.6 mg l^{-1} until day 50, indicating that the biofilm was active in spite of not degrading all the 4-CP supplied to the reactor. After decreasing the 4-CP loading to 25 mg l^{-1} (days 52–87), a gradual decrease in the outlet 4-CP concentration was observed, and from day 68 4-CP was no longer detected in the effluent (Fig. 1, B), while the level of 4-CP degraded was maintained at 25.1±3.1 mg l^{-1}. Due to a disruption of the reactor (fractured glass), which occurred at day 75, the colonized GAC was transferred to a new column (same dimensions); care was taken so that the biofilm matrix was disturbed to a minimum. After restarting the feed, 4-CP was detected in the reactor effluent (days 75–87), despite the fact that the biofilm was active during that period, as shown by chloride liberation. Feeding of 4-CP to the reactor was stopped temporarily from day 88 to day 113, during which time 4-CP was undetectable in the effluent (Fig. 1, C). Chloride release continued during this period of suspended feeding,

Table 1 Degradation of 4-chlorophenol (4-CP) in the granular activated carbon (GAC) reactor during continuous operation

Feed period (days)	4-CP influent load (g l^{-1} d^{-1})	4-CP in influent (g)	4-CP in effluent (g)	4-CP degraded (g)	Removal capacity (g l^{-1} d^{-1})
A. 0–51	4.24	5.37	0.73	3.72	2.89[a]
B. 52–87	2.12	1.86	0.46	2.02	2.27
C. 88–113	0.00	0.00	0.03	0.63	0.99
D. 114–166	2.12	2.84	0.47	1.95	1.47
E. 167–200	1.69	1.28	0.08	1.24	1.47
0–200		11.35	1.76	9.55	

[a] The values for period A refer only to days 14–51

a result indicating that 4-CP adsorbed to the GAC could be assimilated by the biofilm community. Between days 114 and 166, 4-CP was reloaded on the column at a concentration of 25 mg l^{-1}. 4-CP was detected in the effluent at concentrations varying between 0–7.7 mg l^{-1}. Chloride release showed an average concentration of degraded 4-CP of 15.3±2.0 mg l^{-1}. 4-CP concentration in the outlet decreased to an average level of 1.2±0.5 mg l^{-1} when the influent concentration was reduced to 20 mg l^{-1} (days 167–200); 4-CP was not detected in the effluent at several sampling points (Fig. 1, E). From day 167 until the last day of operation, chloride liberation indicated a 4-CP degradation of ca. 16.6±2.0 mg l^{-1}.

The quantity of 4-CP degraded during each phase of the biotreatment is shown in Table 1. During the feeding period between days 52 and 87, total 4-CP degradation was higher than the amount supplied for that period, indicating that 4-CP previously adsorbed to the column was being biodegraded. Of the total amount of 4-CP supplied to the reactor, approximately 85% was biodegraded. The activity of the biofilm was highest during the first half of bioreactor operation, as shown by the average values for removal capacity (Table 1).

During the continuous flow operation of the reactor, the detachment of bacteria from the GAC particles into the effluent was monitored. Samples were taken every month and values between 1.4×10^6 cfu ml^{-1} and 2.0×10^7 cfu ml^{-1} were observed. The organisms present in the effluent were not characterized.

Biofilm activity after 4-CP starvation

At the end of the continuous flow operation the biofilm reactor was starved of 4-CP for 5 months, after which the degrading capacity of the biofilm was tested both under recirculating and continuous flow modes. During the recirculating regime, 4-CP was fed twice to the reactor, and chloride release was immediately observed (Fig. 2), indicating that the biofilm consortium was still exhibiting 4-CP degrading activity. The levels of chloride release also indicated that most of the 4-CP was degraded (ca. 85%). The fact that 4-CP was not detected in the feed recirculation vessel, while chloride was released, suggested that biodegradation occurred after 4-CP adsorption to the GAC. After switching to continuous

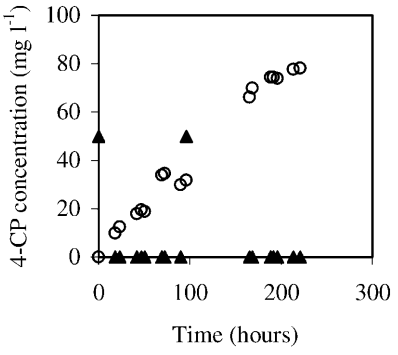

Fig. 2 Degradation of 4-chlorophenol (4-CP) in the biofilm reactor after the starvation period (recirculating operation mode). *Black triangles* 4-CP concentration in the recirculating vessel, *open circles* 4-CP degraded based on chloride release

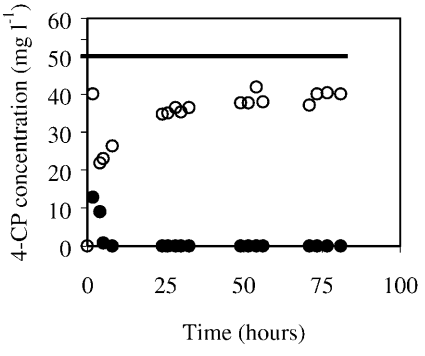

Fig. 3 Degradation of 4-chlorophenol (4-CP) in the biofilm reactor after the starvation period (continuous operation mode). *Horizontal bar* 4-CP concentration in the feed, *open circles* 4-CP degraded based on chloride release, *black circles* 4-CP in the effluent

flow mode, 4-CP degradation stabilized after 20 h, as indicated by chloride release (Fig. 3). The presence of 4-CP was only detected at low levels in the reactor effluent in the first 8 h. Chloride release data indicated that 78% of the total amount of 4-CP added during continuous mode was biodegraded, while 1% was recovered at the outlet of the reactor. The activity of the biofilm recovered completely after 4-CP starvation; the removal capacity was ca. 3.34 g l^{-1} day^{-1}, which accords closely with the values initially obtained for the continuous operation (Table 1).

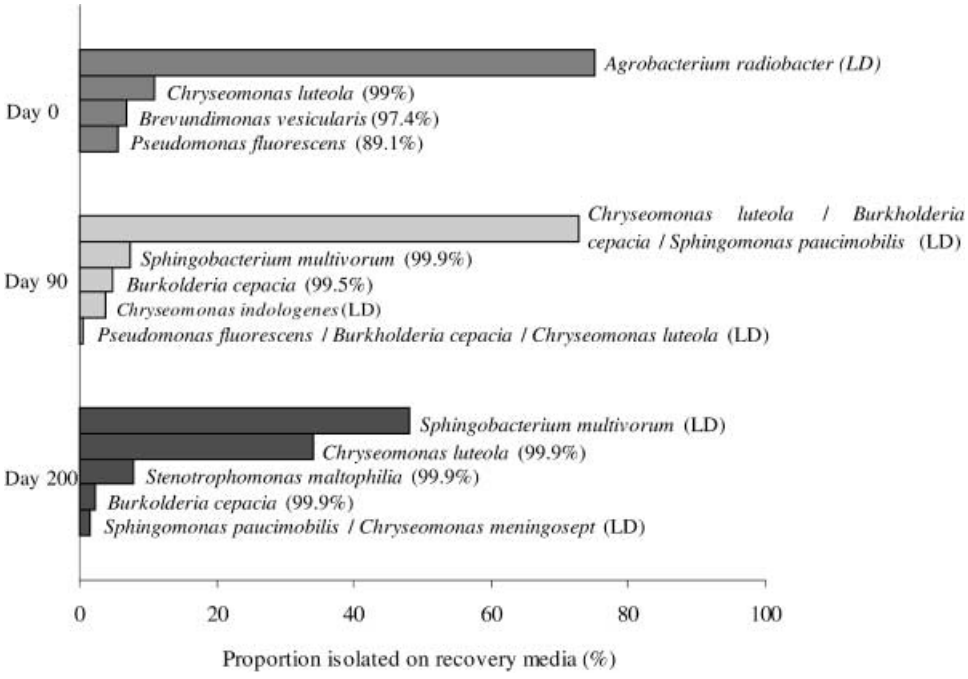

Fig. 4 Characterization of the bacteria consortia isolated from the biofilm reactor throughout the continuous flow operation, recovered in NA. The values in parenthesis show API identification probability (LD – low discrimination)

Analysis of the biofilm community

GAC biomass loading was determined at different stages of continuous flow operation. The bacterial population sizes adsorbed onto GAC at days 0, 90 and 200 were $1.01\pm0.3\times10^8$ cfu g^{-1} GAC, $2.09\pm0.6\times10^9$ cfu g^{-1} GAC and $1.65\pm0.2\times10^9$ cfu g^{-1} GAC, respectively. The bacteria recovered from the GAC reactor were investigated for their 4-CP-degrading capacity; 50 mg l^{-1} 4-CP was degraded within 24–29 h, in batch cultures.

During continuous flow operation of the reactor, a preliminary characterization of the biofilm microbial community was made. Five different morphological colony types were obtained from GAC at the beginning of the continuous flow period, three were recovered on NA, one on minimal salts medium and one in both media. Preliminary characterization of the five bacterial strains showed that all were Gram-negative rods. The isolates were identified by the API 20 NE system and the results for the ones recovered in NA are shown in Figure 4. Due to the small size of the colonies obtained on minimal salts medium agar, bacterial counts were not reliable. The isolation of the morphologically different colonial types recovered on that medium and identification by API revealed the presence of two different bacteria: *Burkholderia cepacia* (identification probability: 99.5%) and *Agrobacterium radiobacter* (identification probability: 95.7%), which was also recovered on NA medium. At the mid-stage of the continuous experiment (day 90), five Gram-negative, rod shaped, bacterial strains were recovered in NA (Fig. 4). In minimal salts medium, two types were recovered, identified by API 20 NE system as *B. cepacia* (identification probability: 99.5%) and *Sphingobacterium multivorum* (identification probability: 99.9%), which were also present on NA. Characterization of the bacteria extracted at the end of the continuous experiment (day 200) and recovered in NA revealed five Gram-negative rods (Fig. 4). Visual observation of the bacterial populations recovered in the effluent during the first 30 days of continuous operation showed mainly four morphologically different colonial types, with a predominance (65–90%) of a morphological type similar to the one identified as *A. radiobacter* in the initial GAC extracts. From then on, four to five morphologically different colonial types were recovered at each sampling stage, with a predominance (55–85%) of a morphological type similar to the one identified as *S. multivorum* in the subsequent GAC extracts.

After the starvation period, bacterial communities of the biofilm were also extracted from the GAC particles and enumerated. Bacterial populations were found in the order of $1.4\pm0.3\times10^7$ cfu g^{-1} GAC, a lower value than during continuous flow operation. Seven morphologically different organisms were recovered on NA media and two were recovered on minimal agar plates supplemented with 4-CP. These organisms were not characterized.

The degradation of 4-CP by individual species was tested in liquid medium. In control experiments, without inoculation, no chloride release was observed. Only one organism, identified by the API 20 NE system with low discrimination between *Chryseomonas luteola*, *B. cepacia* or *Sphingomonas paucimobilis*, was capable of degrading 4-CP in a mono-species culture at 4-CP concentrations of 25 and 50 mg l^{-1}. In the latter case, 4-CP was completely biodegraded after 30 h.

Discussion

Microbial populations in the rhizosphere are one to two orders of magnitude larger than those in adjacent soil

without roots (Whipps and Lynch 1986). Also, they tend to have faster growth rates and to differ in their nutritional requirements and metabolic capabilities, including their abilities to degrade xenobiotics (Siciliano and Germida 1999; Chaineau et al. 2000). Based on these facts, rhizosphere soil samples from a contaminated site were investigated as a source of haloaromatic-degrading microorganisms. The dispersion-differential centrifugation technique was used in order to maximize the diversity of microorganisms to be used as the enrichment inoculum in the reactor. The use of phenol and 4-CP as the enrichment substrates for the development of a 4-CP-degrading biofilm population was successful. 4-CP has been shown to be degraded as a co-metabolite in the presence of phenol (a more easily biodegradable compound) by *A. eutrophus* (Hill et al 1996). However, in addition, phenol stimulates the degradation of other aromatic compounds such as *o*-cresol, *p*-cresol and methylphenols (Arcangeli and Arvin 1995) and thus, in the present study**,** phenol was added initially to stimulate growth and possibly to increase the diversity of microorganisms. As confirmed by the degradation tests made using the GAC bacterial extracts, 4-CP was used as a sole source of carbon by the enriched consortium, thus the addition of phenol was stopped.

Lee et al. (1994) reported that phenol and chlorinated phenols are more effectively biodegraded by immobilized rather than suspended bacteria. Degradation of matrix-bound chlorophenols by fungi has also been reported. Removal of di- and penta-chlorophenol from aqueous solutions treated by cultures of *C. versicolor* has been shown to improve when the fungus was immobilized onto compact matrixes based on wheat bran and husk bran; the effect was attributed to the more efficient production of laccase by an established biofilm, but also to the binding of the chlorophenols onto the surface of the matrixes, allowing a longer exposure to laccase on the surface and a faster removal rate (Ullah et al, 2000). We were interested in evaluating the performance of a GAC biofilm reactor for the degradation of 4-CP under continuous flow mode operation. Biodegradation of 4-CP was achieved at feeding concentrations between 0 and 50 mg l^{-1}. Chloride release values indicate that ca. 85% of the total 4-CP loaded to the column was biodegraded by the biofilm. It is possible that incomplete degradation of the compound and repolymerization phenomena could have occurred within the biofilm, but this possibility was not investigated. However, the growth of the biofilm, accompanied by continuous liberation of bacteria to the column effluent, suggested that the compound was mineralized. In addition, the bacteria recovered from the column at different operational stages demonstrated growth and stoichiometric liberation of chloride in batch cultures supplied with 4-CP.

4-CP was also detected in the column effluent at different stages of bioreactor operation, and there was a tendency for the residual concentration of 4-CP to increase during two of the continuous regimes (Fig. 1, A, D), suggesting that the adsorption capacity of the activated carbon was either exhausted or was not compensating for the 4-CP that was not being biodegraded during its passage through the reactor. During the first phase, when 4-CP was fed at the highest concentration of 50 mg l^{-1}, the rate of dechlorination was relatively high from day 14 to 51, for which period the highest removal capacity was observed. At this 4-CP concentration, oxygen limitation throughout the length of the reactor could have occurred and reductive dechlorination within anoxic niches may be considered, although no attempts were made during the study to recover 4-CP-degrading strains under anaerobic conditions. Also, the bacterial consortia recovered at each stage were always capable of aerobic degradation of 4-CP. Reductive dechlorination may have contributed to 4-CP degradation, and could have led to the production of phenol, although it was never detected at the column outlet. At the lowest 4-CP concentration, the oxygen available in the feed should be sufficient to maintain aerobic conditions in the reactor, and it is interesting to note that the removal capacity remained approximately constant throughout the last two phases of operation (85 days). The appearance of 4-CP in the column outlet during bioreactor operation could also be due to compaction of the GAC bed or to excessive growth of biomass, causing restriction to the medium flow and the formation of channels within the GAC bed. Excessive growth could lead to the clogging of the reactor and, consequently, to decreases in the biofilm activity because the development of anaerobic and dead zones may not contribute to 4-CP biodegradation due to poor contact with the influent, or to oxygen debt. The increase in biomass loading observed during the operation of the bioreactor supports such a hypothesis. Clogging problems have also been reported by other authors (Klecka et al. 1996; Weber and Hartmans 1996; Jorio et al. 1998). The performance of our reactor recovered after these situations, especially when the 4-CP concentration was lowered to 20 mg l^{-1}.

In the present study, biofilm bacteria could degrade 4-CP in the influent feedstock and also 4-CP adsorbed onto the GAC matrix. The latter feature was particularly visible in the period when 4-CP was not fed to the reactor but chloride release continued to be observed. Several hypotheses have been raised concerning to the bioavailability of the adsorbed compounds. The hypothesis of desorption by exoenzymatic reactions has been suggested as the basis for bioregeneration mechanisms (Perrotti and Rodman 1974). However, Schultz and Keinath (1984) indicate that sorbed compounds are utilized through the reversibility of adsorption. Shi et al (1995) demonstrated that bioregeneration occurred in biological activated-carbon systems fed with toluene. Bioregeneration of packed-bed GAC reactors loaded with 4-CP has been previously reported from our laboratories (Caldeira et al. 1999).

The 4-CP degrading biofilm consortium was capable of surviving a starvation period of at least 5 months, a result that reinforces the stability claimed for this kind of biotreatment system. The fact that the biofilm remained

capable of 4-CP degradation suggests that biofilms are able to maintain active populations of competent microbial strains, even when the specific feedstock is intermittently removed. Starvation trials also have been made by other authors, although over much shorter periods of time. Jorge (2000) reported a 10-day period of starvation in a suspended culture, after which no change in the activity of the microbial culture responsible for toluene biodegradation was observed. Hekmat et al. (1997) applied cycling starvation periods of 8 h a day, 7 days a week, to immobilized microorganisms, and no significant change in the average degradation rate of polyalkylated benzenes was observed. In addition, the latter author reported a significant increase in the microbial degrading activity through the cyclical starvation periods. This latter feature agrees well with findings reported by other authors (Subrahmanya Sastry et al. 1994; Kaya et al. 1996; Watanabe et al. 2000). Survival under non-operating conditions may be an important factor for biotreatment operations as starvation periods are a common scenario found in the industrial environment resulting from interruptions of the industrial process leading to periods with no effluents to be treated.

It is clear from the results of the continuous flow operation that the biofilm populations were dynamic as a function of time (Fig. 4). One contribution to this feature may be the development of other competent degrading species, as a result of the selective pressure created by the 4-CP. Biofilms are always subjected to interactions such as symbiosis, or competition for space, or common substrates, thus it is not surprising that dynamic changes of biofilm populations occur in these systems. The microbial characterization of the GAC biofilm revealed the presence of one common bacterium, present as a small proportion (<6%) of recovered bacteria, at all sampling stages during operation – *B. cepacia* (Fig. 4). *B. cepacia* was also recovered in minimal salts medium agar. Degradation of diverse halo-organic compounds by *Burkholderia* species has been reported by several authors (Seeger et al. 1999; Johnson et al. 2000; Nishino et al. 2000), although our isolate was not capable of degrading 4-CP. Among the biofilm isolates, one bacterium was capable of 4-CP degradation when grown as a monospecies culture; this isolate was identified by the API 20 NE system as presenting low discrimination between *C. luteola*, *B. cepacia* or *Sphingomonas paucimobilis*. It is interesting to note, that this isolate dominated the consortium at the time of the second biofilm analysis (day 90), but it was not recovered at the other sampling stages. It is possible that this strain dominated the biofilm consortium during the first stages of operation, when the 4-CP concentration was higher but, after longer exposures to lower concentrations, the strain could have decreased in proportion or may have been lost from the biofilm. In any case, this fact suggests that biodegradation may be performed in a cooperative fashion by the biofilm bacteria, which may vary in composition accordingly to fluctuations in the feed. In addition, this may indicate that typical degrading laboratory species may fail to implant successfully within treatment populations, compared to bacterial communities established naturally in biofilms. The degradation of 4-CP by single species has also been reported by other authors (Bae et al. 1996; Kafkewitz et al. 1996; Hollender et al. 1997; Finkel'shtein et al. 2000).

The results described in this paper showed that the biofilm reactor was capable of degrading different concentrations of 4-CP in an aqueous feed, through a combination of adsorption and biodegradation, leading to a further understanding of bioreactor behaviour under continuous operation for an extensive period of time. The lower efficiencies observed at some stages during the 6-month operation were probably due to oxygen debt within the biofilm and/or biomass clogging, which may be overcome with more efficient aeration of the bed and by devising strategies to prevent clogging and formation of preferential channels. Despite that, bacterial activity continued throughout the operation, although removal capacity was higher during the first 3 months. The biofilm communities established on the GAC seemed to be dynamic, which on one hand may be related to the higher stability claimed for biofilm systems but, on the other hand, calls for attention when introducing laboratory species into this kind of biotreatment system. The stability of such systems is reinforced by the survival of the GAC biofilm consortium of a starvation period of 5 months, which can be a major factor in selecting a biotreatment process for industrial environments where such perturbations are common.

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